Increased mitogenic activity of scleroderma serum: inhibitory effect of human recombinant interferon-gamma

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Abstract

Objectives—To investigate the role of platelet activation in the development of systemic sclerosis and the role of interferon-gamma (IFN γ) in the inhibition of mitogenic activity induced by whole blood serum of patients with systemic sclerosis.

Methods—The mitogenic activity of whole blood serum in the absence or presence of different concentrations of IFN γ (a potent inhibitor of induced collagen synthesis in dermal fibroblasts) and platelet-poor plasma derived serum were tested on human dermal fibroblasts by measuring incorporation of [3 H]thymidine. Platelet activation was determined by quantification of plasma β -thromboglobulin (β -TG) using a β -TG radioimmunoassay kit.

Results—The mitogenic activity was significantly increased in whole blood serum and in platelet-poor plasma derived serum of the patients compared with controls. In contrast, no significant increase in β -TG concentration was observed in scleroderma platelet-poor plasma compared with control. Recombinant human IFN γ had a greater inhibitory effect on the mitogenic activity induced by whole blood serum of patients than on that produced with control sera, at any concentration of IFN γ tested.

Conclusions—Our results suggest that mitogenic activity observed in the plasma of sclerodermic patients could originate from cells other than platelets and could be involved in the development of fibrosis. The potent inhibitory effect of IFN γ on this proliferative activity may account for the beneficial effect of this cytokine in the treatment of progressive systemic sclerosis.

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measured on a fibroblast cell line, NIH 3T3, has been reported in patients with SSc. In contrast, inhibition of the endothelial cell proliferation and a cytotoxic effect induced by scleroderma serum have been reported.¹⁻³

An abnormal proliferation of skin fibroblasts and accumulation of collagen in the connective tissue matrix suggest that growth factors such as platelet derived growth factor (PDGF) or transforming growth factor- β (TGF β) could be involved.^{4,5} This has been established by immunohistological detection of PDGF in the capillaries, mononuclear infiltrates, and fibrotic dermal stroma of scleroderma skin lesions.⁶ Moreover, circulating cells such as monocytes and platelets can be activated after subendothelial damage and release PDGF, TGF β , or both. In addition, PDGF receptor expression is increased in the skin of patients with systemic sclerosis and this suggests that the release of PDGF may be enhanced at sites of PDGF receptor overexpression.⁷ However, plasma PDGF concentrations in patients with scleroderma have been shown to be not significantly different from those observed in normal controls.⁸

In vitro recombinant interferon-gamma (IFN γ) inhibits the mitogenic effect of PDGF on human foreskin fibroblasts AG1523.⁹ IFN γ also reduces collagen synthesis and procollagen mRNA concentrations in normal and scleroderma fibroblasts stimulated by 2% fetal bovine serum, and modulates fibronectin production.^{10,11} Because the increased mitogenic activity of scleroderma plasma remained controversial, we measured the mitogenic response of human dermal fibroblasts to whole blood serum or platelet-poor plasma derived serum from scleroderma patients and from control subjects, and tested the effect of IFN γ on this proliferative response.

Patients and methods

PATIENTS

We studied eight patients with clinical symptoms either progressive systemic sclerosis (PSS) (n = 3) or CREST syndrome (n = 5) of 5-28 years duration. The table summarises their main clinical and biological features; all satisfied the American Rheumatism Association preliminary criteria for the classification of their disorder as systemic sclerosis (SSc).¹² All patients had Raynaud's phenomenon and six of them had also a history of digital ulcers. Visceral involvement (dysphagia, pulmonary fibrosis or Sjögren's syndrome) was observed in three

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Systemic sclerosis (SSc) is a connective tissue disease of unknown aetiology, characterised by fibrosis of the skin and vascular organs, the lung, the intestinal tract, the kidney and the heart, accompanied by proliferation of the vascular intima. The mechanisms responsible for the collagen deposition and the micro-vascular abnormalities are still unknown. Increased production of growth factors has been postulated as a possible mechanism. Thus increased plasma mitogenic activity

Clinical and laboratory features of the patients with scleroderma

Patient No.	Age/sex	Disease duration	Classification	Organ involvement	ANA titre/pattern	ACA	Anti-SCL 70	Platelet count ($\times 10^9/l$)
1	35/F	10	CREST	—	1:10 H	+	—	231
2	59/F	8	CREST	—	1:1000 S	+	—	244
3	61/F	8	CREST	—	1:100 S	+	—	300
4	45/F	10	CREST	—	1:10 S	+	—	298
5	47/F	13	CREST	—	—	+	—	179
6	60/F	5	PSS	Pulmonary, skin, and oesophageal fibrosis	1:10 000 H	—	+	268
7	58/F	5	PSS	Facial skin, oesophageal and pulmonary fibrosis	1:100 S	—	+	302
8	58/F	28	PSS	Pulmonary fibrosis, Sjögren's syndrome, oesophageal sclerosis	1:10 000 H 1:1000 N	—	+	177

ANA = Antinuclear antibodies; H = homogeneous; S = speckled; N = nucleolar; ACA = anticentromere antibodies; CREST = partial or complete CREST syndrome, PSS = progressive systemic sclerosis.

patients. Only one patient had no positive antinuclear antibodies. Anticentromere antibodies were present in the five patients with limited cutaneous involvement, and SC170 antibodies were detected in the three others in association with diffuse scleroderma with visceral organ involvement. Capillaroscopy showed the presence of megacapillaries in all patients. Platelet counts were in the range $177\text{--}302 \times 10^9/l$, compared with $200\text{--}300 \times 10^9/l$ in control samples obtained from healthy volunteers aged between 26 and 40 years. No patient received aspirin or non-steroidal anti-inflammatory drugs.

BLOOD SAMPLE

After venous puncture without anticoagulant, serum was obtained by clotting at 37°C for three hours. For platelet-poor plasma (PPP) derived serum, blood was collected into 3.8% sodium citrate (1:9 v/v) and PPP obtained by centrifugation for 15 minutes at 3000 g, then platelet-poor derived serum was prepared by clotting PPP after recalcification with $20 \mu\text{mol/l}$ CaCl_2 at 37°C for three hours.

MITOGENIC ACTIVITY

Mitogenic activity of whole blood serum and PPP-derived serum was assayed by their ability to stimulate incorporation of [^3H]thymidine (TdR) into human dermal fibroblasts. Cells were seeded in 12 well plates (Costar, Cambridge, USA) in minimum essential medium with 20% fetal calf serum (FCS). Three days later, fibroblasts were deprived for 48 hours under Ham's F 12 medium with 0.5% FCS. Increasing concentrations of blood serum with $\text{IFN}\gamma$ 0–100 U/ml (gift of Boehringer Ingelheim Laboratories, Reims, France) or PPP-derived serum from patients and controls were simultaneously added in the presence of [^3H]TdR $1 \mu\text{Ci/ml}$ for 48 hours at 37°C . After precipitation with 10% ice-cold trichloroacetic acid, cells were washed and solubilised with 0.2 mol/l NaOH. Radioactivity of lysates was measured with a betacounter.

 β -THROMBOGLOBULIN ASSAY

Plasma was chilled on ice as soon as it was obtained from patients and controls, and stored at -20°C . Concentrations of β -TG were assayed using a β -TG radioimmunoassay kit (Amersham, Les Ulis, France).

STATISTICAL ANALYSIS

Data are expressed as mean (SEM). Comparisons were performed with the Mann-Whitney non-parametric test.

Results

MITOGENIC ACTIVITY INDUCED BY SCLERODERMA SERUM

Fibroblast mitogenic activity, measured by incorporation of [^3H]TdR, was significantly greater with all concentrations (5–20%) of total blood serum in the eight scleroderma patients than in controls, but the two subgroups of scleroderma patients did not differ in the mitogenic activity of their sera (fig 1A).

Platelet-derived serum from scleroderma patients had a greater stimulatory effect on fibroblast proliferation than that from controls, for each concentration tested. This stimulatory effect increased in a concentration dependent manner in the scleroderma patients, while the controls did not show significant changes (fig 1B).

Figure 1 Effect of different concentrations of whole blood serum (A) and platelet-poor plasma derived serum (B) in patients with progressive systemic sclerosis (PSS, $n = 3$) or CREST syndrome ($n = 5$) and in control subjects ($n = 6$). Results are the mean (SEM) of three different experiments. * $p = 0.03$; ** $p = 0.01$; *** $p = 0.006$ compared with control.

β -THROMBOGLOBULIN PLASMA CONCENTRATION
To determine if the growth factor activity that we found in the platelet-poor plasma derived serum was related to an intravascular platelet activation, we measured the plasma concentration of β -TG, a specific α granule protein released in the circulation by activated platelets. No significant increase of β -TG was observed in scleroderma platelet-poor plasma (37 (18) ng/ml) compared with the controls (36 (19) ng/ml).

EFFECT OF IFN γ ON SCLERODERMA SERUM-INDUCED MITOGENIC ACTIVITY

At 10% serum concentrations, all scleroderma sera had a more potent fibroblast mitogenic activity than those from controls. For each patient, IFN γ 10–100 U/ml inhibited [3 H]TdR incorporation induced by 10% serum in a dose dependent manner, and to a greater extent in scleroderma patients than in controls (fig 2).

Discussion

Involvement of activated platelets in scleroderma is still controversial. Recently measurements of PDGF and β -TG by immunoassay in the plasma of patients with scleroderma showed no difference between normal controls and patients with RA.⁷ A growth inhibitory and a cytotoxic effect of SSc plasma have been reported in the literature on endothelial cells.^{1–3} In this study we observed that the mitogenic activity induced by 10% of whole blood derived serum from patients with scleroderma was significantly greater than that induced by serum from controls. However, an increased mitogenic activity with small (2.5%) rather than large concentrations has been shown in blood serum of scleroderma patients, and the authors¹³ suggested that TGF β could originate from platelets and other blood cells and have an inhibitory role. This hypothesis is in contradiction with another study showing no significant difference of TGF β expression in SSc.⁶ Moreover, we did not find platelet activation when we measured β -TG in plasma from scleroderma patients, suggesting that TGF β

present in platelets was not involved. This is in agreement with recent published data.⁷

In the present study, PPP derived serum from patients exhibited an increased mitogenic activity depending on the concentration used, while no modification of the mitogenic activity was observed with PPP derived serum from controls. No correlation could be established between extensive cutaneous fibrosis and plasma mitogenic activity; sera from SSc patients (patients 6 and 7), for example, did not exhibit increased mitogenic activity for dermal fibroblasts compared with sera from others. In two patients who presented with extensive skin fibrosis, no correlation between fibrosis and greater mitogenic activity was observed. These data are similar to those obtained by Pandolfi *et al*¹³ with the cell line NIH 3T3. We propose that PDGF or other growth factors present in platelets did not play a role in mitogenesis induced by platelet-poor plasma derived serum. Mitogenic activity induced by PDGF and other growth factors can be secreted from other cells than platelets; PDGF has been detected by immunohistological techniques in the endothelial lining of small capillaries, and in macrophage cytoplasm.⁶

It has been shown that IFN γ can be effective in the treatment of patients with SSc.¹⁴ After a six month treatment period with recombinant human (rh) IFN γ , a significant improvement was observed in 10 patients, in particular in the skin fibrotic abnormalities. In addition, in vitro studies have showed an inhibition of excessive scleroderma fibroblast collagen production by (rh)IFN γ .^{10–11} We also have found that (rh)IFN γ is a potent inhibitor of scleroderma serum induced mitogenesis in fibroblasts. All the scleroderma sera tested exhibited increased mitogenic activity along with inhibition by IFN γ (60–79%) compared with normal sera (46–64%). Other interferons, alfa and beta, have been shown to inhibit collagen synthesis and dermal fibroblast proliferation in vitro; the suppressive effect of IFN α procollagen mRNA was somewhat weaker than that of IFN γ .¹⁰ IFN α has already been used in a pilot study of the treatment of diffuse cutaneous systemic sclerosis;¹⁵ the cutaneous disease was stabilised or improved in 64% of patients, but no significant effect was observed on other functional or clinical parameters.

Our results are in agreement with the more striking improvement observed with IFN γ therapy in skin fibrosis than was found for visceral involvement in vivo. As fibroblast dermal proliferation induced by mitogenic activity is involved in diseases such as scleroderma, IFN γ may provide a rational therapeutic approach.

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Figure 2 Effect of different concentrations of IFN γ on mitogenic activity ([3 H]TdR incorporation) induced by 10% blood serum from patients with progressive systemic sclerosis (PSS, $n = 3$) or CREST syndrome ($n = 5$) and control subjects ($n = 6$). Results are the mean (SEM) of three experiments.

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